Some properties of membrane-bound, solubilized and reconstituted into liposomes H^+-ATPase of vacuoles of *Saccharomyces carlsbergensis*

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Vacuoles of yeast grown in peptone medium possessed high ATPase activity (up to 1 μmol · mg protein^{-1} · min^{-1}). Membrane-bound and solubilized ATPase activities were insensitive to vanadate and azide, but were inhibited by NO_3^-. K^+ and cyclic AMP stimulated both membrane-bound and solubilized ATPase activities. Dio-9 activated the membrane form of vacuolar ATPase 1.5–2-fold and did not affect the solubilized enzyme. Solubilized and partially purified vacuolar ATPase was reconstituted with soy-bean phospholipids by a freeze-thaw procedure. ATPase activities in native vacuoles and proteoliposomes were stimulated effectively by Dio-9, the protonophore FCCP and ionophores valinomycin and nigericin. ATP-dependent H^+ transport into proteoliposomes was also shown by quenching of ACMA fluorescence. Vacuolar and partially purified ATPase preparations possessed also GTPase activity. Unlike ATPase, however, GTPase was not incorporated as a proton pump into liposomes.

ATPase *GTPase* *Solubilization* *Purification* *Reconstitution* *Liposome* *H^+ transport*

1. INTRODUCTION

Several groups have reported the existence of an electrogenic ATP-dependent H^+ pump in vacuoles of yeast [1] and plant [2–4] cells. The available data suggested the pump to be H^+-ATPase, energizing the secondary transport of substances across the tonoplast [3–7]. The isolation and partial purification of ATPase from vacuoles of the yeast *Saccharomyces carlsbergensis* [8] was the direct proof of the tonoplast possessing its own ATPase, which was distinguishable from plasmalemma and mitochondrial ATPases. To show that vacuolar ATPase drives the electrogenic H^+ transport it was necessary to incorporate the solubilized enzyme into liposome membranes in a functionally active form. Reconstitution of the anion-sensitive H^+-ATPase solubilized from the microsomal fraction of corn roots and identification with the tonoplast ATPase have been reported [9]. The reconstitution was accomplished by gel filtration of deoxycholate-solubilized ATPase through Sephadex G-200 in the presence of phospholipids. The ATPase was not purified previously.

Here we describe some properties of membrane-bound, solubilized H^+-ATPase of vacuoles isolated from yeast, purified and reconstituted into liposomes.

Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Zwittergent TM-314, 3-(tetradecyldimethyl ammonium)-1-propanesulfonate; DTT, dithiothreitol

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2. MATERIALS AND METHODS

S. carlsbergensis IBPhM-366 was grown in medium containing 1% peptone, 0.5% yeast ex-

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tract and 2% glucose. To obtain spheroplasts 30 g exponentially grown cells were suspended in 240 ml medium A (0.7 M mannitol, 1.5% lyophilized snail gut juice, 10 mM DTT, 0.14 M Na-citrate, pH 6.7). The cell suspension was incubated for 50 min at 30°C. The spheroplasts obtained were washed with solution A without snail gut juice and DTT.

To isolate the vacuoles the spheroplasts were exposed to osmotic shock by suspension in 4% Ficoll prepared in medium B (10 mM Na-citrate, pH 6–8; 0.1 M sorbitol). The spheroplast homogenate (15 ml) obtained was transferred to centrifuge test tubes, and 15 ml of 2% Ficoll in medium B followed by 8 ml medium B were gently layered on top. After 30 min centrifugation at 25 000 rpm (Beckman SW-27 rotor) vacuoles were found at the boundary of the 2% Ficoll and solution B layers. The vacuolar suspension was diluted 4 times with medium B and vacuoles were sedimented by centrifugation of the suspension for 50 min at 3500 × *g*.

Vacuole membrane-bound ATPase was solubilized by Zwittergent TM-314 (1 mg/mg protein) and then purified in a glycerol density gradient [8]. To reconstitute the ATPase into liposomes freeze-thawing was used [10]. Soy-bean phospholipids (40 mg) were suspended in 1 ml medium containing 10 mM Pipes–KOH (pH 6.0), 3 mM mercaptoethanol, 1 mM EDTA, 25 mM K_2SO_4. The suspension was sonicated at 4°C in an MSE (England) ultrasonic desintegrator. The preparation of partially purified ATPase was added to liposomes (0.2 mg phospholipids/μg protein) and rapidly frozen in a dry ice–acetone mixture. After thawing at room temperature, the preparation was used for analyses.

Phosphohydrolase activities were determined at 30°C after 10 or 20 min incubation in 10 mM Mes–NaOH (pH 6.5), containing 0.75 mM ATP or GTP (sodium salts), 0.75 mM $MgSO_4$ and 0.1 M sorbitol. Inorganic phosphate was determined as in [11]. Protein was assayed as in [12].

H^+ transport in proteoliposomes was monitored by fluorescence quenching of ACMA [5].

3. RESULTS AND DISCUSSION

Vacuoles isolated from yeast grown in peptone-containing medium had higher ATPase activity in comparison with that of yeast grown in less rich nutrient medium [5]. ATPase activity could be increased by incubation of vacuoles in 150 mM KI. As a result of such treatment 20–40% of protein was lost without decrease in activity and the specific activity increased. This technique was used only before ATPase solubilization.

Table 1

Influence of various compounds on membrane and solubilized forms of vacuolar ATPase

Additions	ATPase activity (%)	
	Membrane	Solubilized
None	100[a]	100[a]
50 μM orthovanadate	100	115
5 mM azide	105	100
50 mM $NaNO_3$	65	29
50 μM cyclic AMP	109	118

[a] Specific activities of membrane and solubilized ATPase were 905 and 4100 nmol $P_i \cdot mg\ protein^{-1} \cdot min^{-1}$, respectively

ATPase activity of vacuoles was not affected by vanadate and azide (table 1), inhibitors of plasmalemma and mitochondrial ATPase, respectively.

ATPase of yeast vacuoles like ATPase of plant vacuoles [13] was inhibited by NO_3^- with the solubilized enzyme being inhibited to a greater degree than the membrane-bound one (table 1). The inhibitor of plant vacuolar ATPase Dio-9 [2,14] at 5–45 μg/ml did not inhibit but stimulated 1.5–2-fold the activity of membrane ATPase of yeast vacuoles (table 2). Solubilized ATPase was insensitive to Dio-9. One could suppose that Dio-9 increased ion permeability of tonoplast and activated membrane ATPase removing proton control.

Data obtained in [15] favor ionophore action of Dio-9. In accordance with our measurements Dio-9 stimulates ΔpH generation and collapses $\Delta\psi$, i.e., it seems to increase anion permeability of the tonoplast (not shown). Dio-9 does not inhibit vacuolar ATPase of *S.carlsbergensis* but does inhibit H^+-ATPase of mitochondria and plasmalemma of fungi [15,16], thus expanding the opportunities of inhibitor analysis in studies of membrane transport properties.

The stimulation of membrane-bound and solubilized ATPases by cyclic AMP was nearly the same (table 1). Therefore, the stimulation of ATPase is not explained by removal of H^+ control.

Treatment of yeast vacuoles with Zwittergent TM 3-14 led to solubilization of ATPase and other vacuolar proteins. Partial purification of ATPase by centrifugation in a glycerol gradient increased the specific activity of the enzyme preparation 4–10-fold, i.e., 4–10 μmol·mg protein^{-1}·min^{-1}.

ATPase was reconstituted into the liposomal membrane without removal of the detergent. This could explain the 3-fold drop of ATPase activity as a result of the reconstitution. Another explanation is concerned with the peculiarities of enzyme incorporation into the liposome membrane.

If H^+-ATPase was incorporated into the liposome in a functionally active form it should translocate H^+ inside the vesicles. The H^+ electro-chemical gradient created would restrict the enzyme activity. Introduction of membrane permeability modifiers collapsing $\Delta\tilde{\mu}_{H^+}$ into the incubation medium should activate H^+-ATPase. Table 2 shows that ATPase of proteoliposomes was activated in the presence of the protonophore FCCP, collapsing $\Delta\tilde{\mu}_{H^+}$ on the membrane, to nearly the same degree as in native vacuoles. The ATPase activity is stimulated even more by valinomycin, increasing the K^+ permeability of membranes and collapsing the $\Delta\psi$ (inside positive). Nigericin induced H^+/K^+ exchange and collapsed ΔpH, increasing highly the activity of ATPase (table 2).

The ionophores used did not noticeably affect the activity of solubilized ATPase in the absence of phospholipids (table 2), thus indicating that H^+-ATPase of the tonoplast was incorporated into the liposome membrane. The stimulation of proteoliposome ATPase by Dio-9 was almost the same as that of the vacuolar enzyme (table 2).

Our results demonstrate that ATP hydrolysis by proteoliposomes is coupled with the translocation of H^+ into the vesicles. This conclusion was supported by measurements of ΔpH. The addition of Mg-ATP to proteoliposomes, containing tonoplast ATPase, resulted in quenching of ACMA fluorescence, indicating acidification of the intravesicular space (fig.1). As expected, the formation of ΔpH was insignificant in the absence of valinomycin (trace 2) as ATPase was apparently blocked by a membrane potential (positive inside). Valinomycin decreasing $\Delta\psi$ stimulates sharply the formation of ΔpH, which is easily collapsed by FCCP or nigericin (fig.1). *S.carlsbergensis* vacuoles possessed besides ATPase activity sufficiently high GTPase activity (400–600 nmol·mg protein^{-1}·min^{-1}).

After treatment of vacuoles with Zwittergent which was followed by the purification of ATPase, GTPase activity doubled. GTPase of vacuoles was activated by ionophores and Dio-9, though less effectively than ATPase (table 3). However, these compounds did not stimulate GTPase activity in either the solubilized or proteoliposome preparation (table 3) indicating that GTPase was not incorporated as an H^+ pump into the liposome membrane. GTPase activity either did not change or decreased by no more than 25% in the process of ATPase reconstitution into liposomes, while ATPase became one third as active. The GTPase

Table 2

Influence of ionophores and Dio-9 on ATPase activity in isolated vacuoles, solubilized preparation of ATPase and proteoliposomes

Additions	ATPase activity (%)		
	Membrane	Solubilized	Reconstituted
None	100[a]	100[a]	100[a]
15 μg Dio-9	170	100	155
K_2SO_4 10 mM	116	117	–
1 μM nigericin + 10 mM K_2SO_4	221	115	250
1 μM valinomycin + 10 μM K_2SO_4	127	90	218
1 μM FCCP	215	102	175

[a]Specific activities of membrane, solubilized and reconstituted ATPase were 1000, 4500 and 1500 nmol P_i·mg protein^{-1}·min^{-1}, respectively

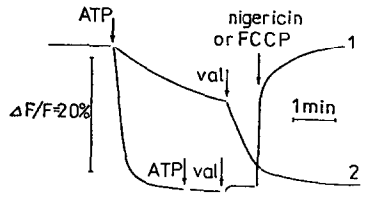

Fig.1. ATP-dependent ΔpH generation across the membrane of proteoliposomes. Incubating medium: 25 mM K_2SO_4, 10 mM Mes-Na (pH 6.0), 34 μg protein and 6.8 mg soy-bean phospholipids, 10 μM ACMA, 0.5 mM Mg ATP. Trace 1: 0.09 μM valinomycin was added before Mg ATP, then the amount of ATP was doubled and valinomycin was added to 0.52 μM. Trace 2: 0.5 mM ATP was added in the absence of valinomycin, then valinomycin (0.43 μM) was added. Nigericin and FCCP, 0.43 μM each.

activity was more stable than that of ATPase if the detergent concentration was increased and after the storage of partially purified ATPase at −5°C. These data and the different distribution of ATPase and GTPase activities at the ATPase purification allow us to suggest that the above mentioned activities are controlled by different enzymes.

The above results of experiments on the reconstitution of tonoplast H^+-ATPase leave no doubt that the enzyme is a proton pump. They show conclusively that the energetics of transport processes through the tonoplast of lower (fungi) and higher plants is based on formation of the electrochemical potential of H^+.

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Table 3

Influence of ionophores and Dio-9 on GTPase activity in isolated vacuoles, solubilized preparation and proteoliposomes

Additions	GTPase activity (%)		
	Membrane	Solubilized	Reconstituted
None	100[a]	100[a]	100[a]
15 μg Dio-9	140	100	100
10 mM K_2SO_4	102	80	–
1 μM nigericin + 10 mM K_2SO_4	137	90	98
1 μM valinomycin + 10 mM K_2SO_4	126	70	80
1 μM FCCP	162	120	102

[a]Specific activities of membrane, solubilized and reconstituted GTPase were 450, 980 and 735 nmol $P_i \cdot mg\ protein^{-1} \cdot min^{-1}$, respectively

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